REMARKS

Claims 1, 4-12, 15-16, 23-32, 34-35, 38-43, 46-59 and 64 were pending as of the date that the Office Action dated November 20, 2003 was received. Herein, claims 1, 4-12, 15-16, 23-32, 34-35, 38-43, 46-59 and 64 are cancelled. New claims 65-89 are added. Support for the new claims can be found in the as-filed specification, e.g., as set forth in the original claims. Additional support for certain claims is discussed, below.

The Examiner extended the courtesy of a telephonic interview to the undersigned on approximately December 9, 2003. The rejections under 35 U.S.C. § 112 ¶ 1 were discussed for the first independent claim. The Examiner's position was, in part, reflected by language set forth in the Office Action at ¶ 4. No agreement was reached. It is believed, however, that the present claims are substantively consistent with the positions that were expressed by the Examiner, except that the present claims do not specify that heparin be present with the bFGF, and the claims have not been limited to Medium I, Medium II, or Medium III.

It is noted that the language set forth in the Office Action at ¶ 4 varies from the disclosed and presently claimed process. Support for the presently claimed process, e.g., independent claim 65, is found in the as-filed specification, e.g., on page 8 under the heading "5.0". The specification describes that astrocytes are cultured in vitro, optionally pretreated with a growth factor, undergo a dissociation step, and are then cultured in the presence of an added factor like bFGF. The cells may then cultured without the added factor, e.g., bFGF. The present claims reflect such a process.

With regards to the inclusion of heparin with bFGF, it is believed that the Examiner has indicated that the addition of heparin is well-known to persons of ordinary skill in the art to be necessary for the function of bFGF. Respectfully, it is submitted that persons of ordinary skill in these arts are aware of many elements necessary for the practice of the claimed methods, e.g., use of an appropriate concentration of CO_2 in the incubator, culturing at physiological pH and

physiological temperature. The claiming of all necessary elements known to persons of ordinary skill is not practical, and, furthermore, unduly limits the claims when unforeseeable future technology replaces such elements, or when such elements are essentially tangential to the practice of the claimed inventions. Therefore the Examiner is requested to allow the claims without reference to the element of heparin. Moreover, if the Examiner is relying on common knowledge in the art, the Applicant respectfully traverses such reliance and requests the Examiner to provide a reference to support this position, as per MPEP 2144.03.

With regards to the type of cell culture medium, it is submitted that a person of ordinary skill in these arts, after reading the present application, will be able to undertake routine experiments as may be needed to optimize the cell culture medium and determinate what variations may be reasonably be made. It is acknowledged that an ordinary artisan would probably acknowledge that the culture of cells can be affected by changes in culture conditions, such as the source of culture water, weather changes, and lot-to-lot variations in supposedly defined media. Nonetheless, the ordinary artisan is able to master such variations in the ordinary course of the practice of these arts, even though such variations may sometimes require experimentation to overcome.

As set forth in the attached article, "Medium Design for Cell Culture processing and Tissue Engineering", as published at http://hugroup.cems.umn.edu/CTRE/cd-rom/Medium%20Design/Medium%20Design.pdf, a person of ordinary skill in these arts is able to determine what cell culture media may reasonably be used. Various factors in the medium may be adjusted and varies so as to best culture a given cell in a particular situation. Factors include energy sources, vitamins, antibiotics, buffers, and various supplements. It is further noted that this article references multiple publications that describe processes for determining suitable medium content, see last three pages therein. Since at least one medium has been disclosed to accomplish the presently claimed functions, routine processes may be used to

develop many variations thereof. Therefore, persons of ordinary skill can be expected to practice variations of the media without undue experimentation. Moreover, limitation of the claims to one very narrow composition of medium for which variations could readily be practiced by ordinary artisans after routine experimentation would unduly narrow the claims so that they would not commensurate with the scope of the invention.

In view of the foregoing, it is submitted that this application is in condition for allowance. Favorable consideration and prompt allowance of the application are respectfully requested.

The Examiner is invited to telephone the undersigned if the Examiner believes it would be useful to advance prosecution.

Respectfully submitted,

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Medium Design for Cell Culture Processing and Tissue Engineering

By Wei-Shou Hu

I. IMPACT OF MEDIA ON THE OVERALL CELL CULTURE PROCESS
II. A GUIDE FOR MEDIUM DESIGN—BODY FLUID
III. BASIC COMPONENTS OF TISSUE CULTURE MEDIUM
- A. WATER
- B. LOW MOLECULAR WEIGHT NUTRIENTS
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  2. NITROGEN SOURCES (AMINO ACIDS)
  3. VITAMINS
  4. BULK IONS
  5. TRACE ELEMENTS
  6. LIPIDS AND PHOSPHOLIPID PRECURSORS
  7. NUCLEIC ACID (RNA AND DNA) PRECURSORS
- C. NON-NUTRIENT SUBSTANCES
  1. ANTIBIOTICS
  2. BUFFERS
  3. PHENOL RED
  4. PROTECTIVE AGENTS
  5. ANTI-OXIDANTS
  6. REDUCING AGENTS
- D. METABOLITES AND CONDITIONING FACTORS
- E. HIGH MOLECULAR WEIGHT FACTORS (SUPPLEMENTS)
  1. SERUM OR BIOLOGICAL FLUIDS
  2. HYDOLYZED PROTEINS

I. IMPACT OF MEDIA ON THE OVERALL CELL CULTURE PROCESS

- Raw material inventory and storage
- Downstream processing
- Bioreactor design and operation
- Cell line stability
- Product yield
- Product quality and assurance
- The overall cost of the final product may depend to a great extent on media formulation and optimization
- Regulatory approval, QC/QA

II. A GUIDE FOR MEDIUM DESIGN—BODY FLUID

Some important constituents and physical characteristics of the extracellular fluid, the normal range of control, and the approximate nonlethal limits

	Normal Value	Normal Range	Approximate Nonlethal Limits	Units
Oxygen	40	35–45	10–1000	mm Hg
Carbon dioxide	40	35–45	5–80	mm Hg
Sodium ion	142	138–146	115–175	mmol/L
Potassium ion	4.2	3.8–5.0	1.5–9.0	mmol/L
Calcium ion	1.2	1.0–1.4	0.5–2.0	mmol/L
Chloride ion	108	103–112	70–130	mmol/L
Bicarbonate ion	28	24–32	8–45	mmol/L
Glucose	85	75–95	20–1500	mg/dl
Body temperature	98.4 (37.0)	98–98.8 (37.0)	65–110 (18.3–43.3)	F° (C°)
Acid-base	7.4	7.3–7.5	6.9–8.0	pH

From Guyton, *Textbook of Medical Physiology, 8th Ed.* (1991) p. 6., W.B. Saunders Co., Pub.

III. BASIC COMPONENTS OF TISSUE CULTURE MEDIUM

A. *WATER*

- Mammalian cells are exceedingly sensitive to the quality of water used for media preparation
- Types of contaminants:
  - Inorganics—heavy metals, iron, calcium, chlorine
  - Organics—by-products of plant decay, detergents
  - Bacteria—endotoxin or pyrogen
  - Particles—colloids or particles
- Contaminants—such as metals, organic materials and endotoxin—can be introduced during water storage
- Water purification methods
  - distillation
  - deionization
  - reverse osmosis (RO)
  - carbon absorption
  - filtration
  - ultra filtration
  - UV irradiation
- It is necessary to use a combination of technologies to reduce contaminants to required levels for critical applications. A typical water preparation process involves filtration, RO or deionization, and distillation.

Water for injection (U.S. Pharmacopeia)

Variable	Requirement
pH	5.0-7.0
Chloride	.05 mg/L
Sulfate	1.0 mg/L
Ammonia	.03 mg/L
Calcium	4.0 mg/L
Carbon Dioxide	5 mg/L
Heavy Metals	1.0 mg/L
Oxidizable Substances	0.8 mg/L
TDS	10 mg/L
Pyrogens	None
Nitrates, Nitrites	Color Test

B. *LOW MOLECULAR WEIGHT NUTRIENTS*

1. ENERGY SOURCES

- six-carbon sugars (5–20 mM)
  - glucose-most common
  - fructose, galactose, mannose, maltose-cell line dependent; may reduce lactate production and good for cell mantenance.
- pyruvate and ribose (eg-uridine)
- Glutamine (1–20mM) (except in glutamine synthetase transfected clones)
  - a major carbon source in most media

- spontaneously decomposes in medium in a time, temperature, serum and phosphate dependent manner (in serum-free DMEM @ 37 °C, t1/2 = 8 days) . A product of glutamine utilization and decomposition is ammonia, a potentially toxic compound

2. NITROGEN SOURCES (AMINO ACIDS)

- Can be divided into essential and nonessential ones based on nutritional studies using tissue culture cells
- Exceptions:
  - proline is required by mutant CHO cells;
  - serine is frequently required at clonal densities;
  - asparagine is required by certain malignant cells;
  - glycine sometimes needed in case of borderline folic acid deficiency or in the presence of falcate analogues-methotrexate and aminopterin
- Quantitative requirement for essential amino acids becomes larger when non-essential ones are not provided
- Hydrolyzates of proteins (peptones), a cheap and satisfactory nitrogen source, have been used to supplement crystalline amino acid mixtures
- Small peptides can serve the same function as amino acids-some of these are more stable (e.g., glycine-glutamine) or get transported more readily than their free amino acids counterparts

Essential and non-essential amino acids

Essential amino acids†	Non-essential amino acids
L-arginine	L-alanine
L-cysteine*	L-asparagine
L-histidine	L-asperatic acid
L-isoleucine	L-glutamic acid
L-leucine	L-glycine
L-lysine	L-proline
L-methionine	L-serine
L-phenylalanine	
L-threonine	
L-tryptophan	
L-tyrosine*	
L-valine	
L-glutamine*	

†For human and albino rat
*Essential for cells in culture

Glutamine Stability
Sigma Cell Culture Technology Catalogue, 1992. Pages 184–186.

TEMP

pH

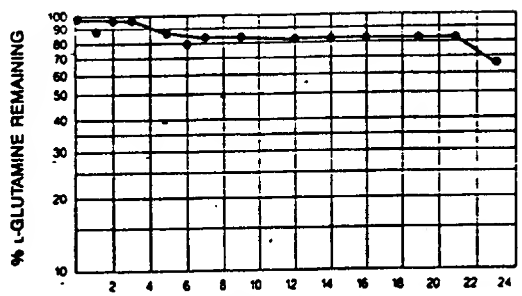


Fig. 1 DAYS IN STORAGE AT 4°C

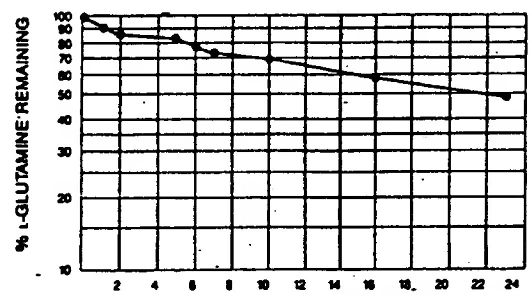


Fig. 4 DAYS IN STORAGE AT pH 3 AND 21°C

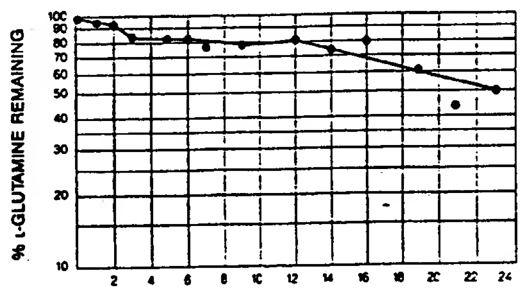


Fig 2 DAYS IN STORAGE AT 21°C

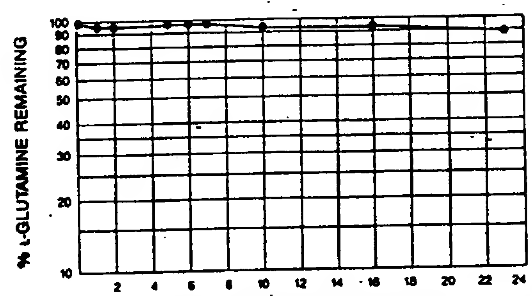


Fig. 5 DAYS IN STORAGE AT pH 7.3 AND 21°C

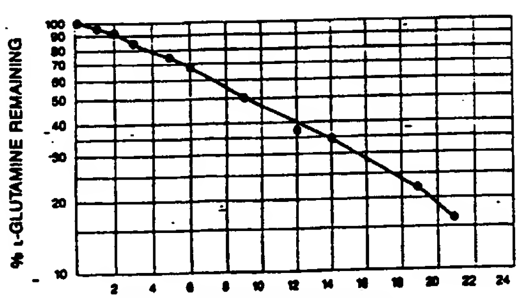


Fig. 3 DAYS IN STORAGE AT 35°C

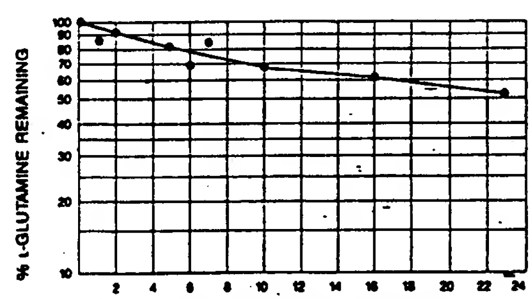


Fig. 6 DAYS IN STORAGE AT pH 9 AND 21°C

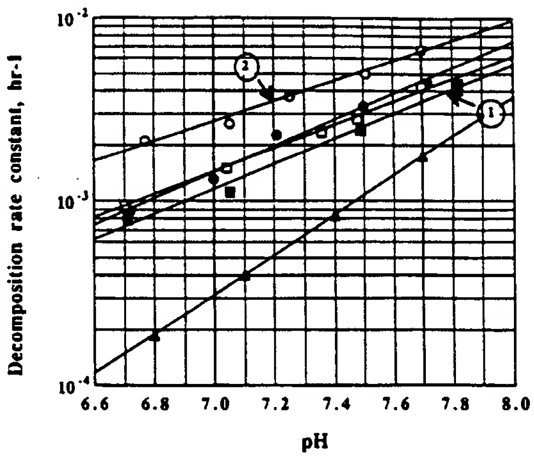
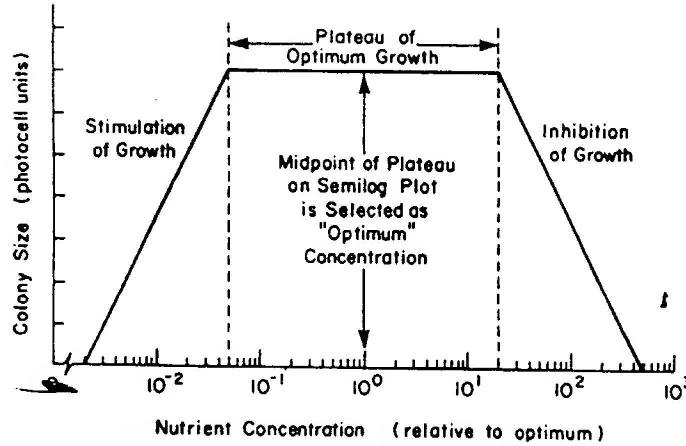
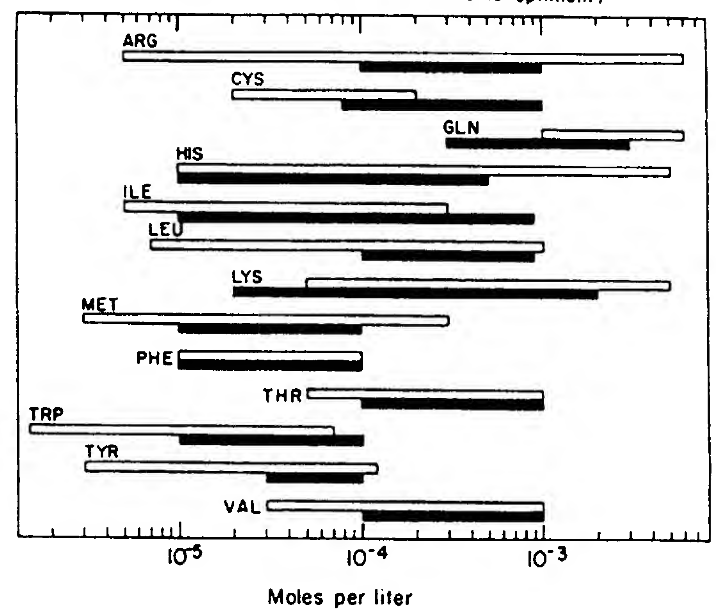


Figure 3. Influence on the decomposition rate constant *k* by pH for the four different media formulations: RPMI-1640 (open circles), IMDM (closed circles), OPTI-MEM (open squares), and DMEM (closed squares). The arrows point to the values obtained by earlier investigators: (1) value of Seaver et al. (1984) in DMEM and (2) value of Trisch and Moore (1962) in PBS. The solid triangles are the data of Lin and Agrawal (1988).



Idealized growth response illustrating the procedure for establishing the "optimum" concentration of a nutrient. A semilog plot of the growth response (linear scale) versus the nutrient concentration (log scale is established).

- The range of optimal concentration for cell growth is wide.



The range of concentrations of essential amino acids required for optimal multiplication of HDF enc. CEF. The growth response of HDF to each indicated amino acid was analyzed in medium MCDB IO5 minus the amino acid under consideration (open bars). The response of CEF to each was analyzed in MCDB 202 minus the single amino acid (solid bars). Each range shown represents the plateau of the curve shown in Figure I determined for each amino acid.

—Ham and Waymouth (1981) The Growth Requirement of Vertebrate Cells in Culture.

3. VITAMINS

- Ascorbic acid may be beneficial for cells that synthesize collagen
- Vitamin A can have a pronounced effects on growth and differentiation of some cell types
- Vitamin K is required for gamma-carboxylation and correct processing of vitamin K dependent proteins
- Vitamin E functions as an anti-oxidant
- Biotin is not present in MEM or DMEM medium, which rely on the serum supplement to provide this vitamin
- Vitamin D regulates Ca^{+2} and is regarded by many as a hormone rather than a vitamin. Most toxic of all vitamins when present in excess.
- Thiamine pyrophosphate catalyses the transfer of carboryl group, transketolase, transaddolase.
- Pyridoxal phosphate (pyridoxine vitamin B_6 catalyses transamination
- Biotin is a carrier of activated CO_2, and is involved in pyruvate dehydugenase, pyruvate carboxylase, fatty acid synthesis
- Cobalmin (B12) is involved in free radical reactions of intramolecular C-C bond rearrangement, methylation, conversion of ribonucleotides to deoxyribosenucleotides.
- These vitamins, although are catalysts and not consumed in reactions, still need to be replenished due to turn over

List of vitamins

Water soluble	Fat soluble
Biotin*	Vitamin A
Folic acid*	Vitamin D
Niacinamide (or nicotinic acid)*	Vitamin E
Pantothenic acid*	Vitamin K
Pyridoxine*	
Riboflavin*	
Thiamin*	
Vitamin B-12*	
Ascorbic adic (vitamin C)	
Lipoic acid	

*Required by cells

Concentrations of bulk ions in basal medium (μM)

	DMEM/F12 (1:1)	William's E	DMEM	RPMI	F12
Na^+	150.31	143.71	155.12	137.74	144.03
K^+	4.18	5.37	5.37	5.37	3.00
Mg^{2+}	0.71	0.81	0.81	0.41	0.60
Ca^{2+}	1.05	1.80	1.80	0.42	0.30
Cl^-	126.66	125.33	118.48	108.03	134.83
PO_4^{3-}	1.02	1.17	0.78	5.63	1.17
HCO_3^-	29.02	26.19	44.04	23.81	14.00
SO_4^{2-}	0.41	0.81	0.81	0.41	0.00
NO_3^-				0.85	0

4. BULK IONS

Roles of bulk ions

- maintenance of membrane potential (Na^+, K^+)
- osmotic balance (Na^+, Cl^-)
- co-factors in various enzymatic reactions
- cell adhesion (Ca^{+2}, Mg^{+2})
- binding of iron to transferrin (HCO_3^-)
- buffering (HCO_3^-, HPO_4^-)

5. TRACE ELEMENTS

- Those clearly required by cultured cells are: iron, manganese, zinc, molybdenum, selenium, vanadium, copper
- Ubiquitous contaminants of chemicals and supplements used in preparation of medium
- Some medium contain rare trace elements such as rubidium, cobalt, zirconium, germanium, molybdenum, nickel, tin and chromium; may be needed for long-term growth in protein-free medium

Trace elements with MCDB 104 (serum-free medium for human diploid cells) (mM)

$CuSO_4 \cdot 5H_2O$*	1.0×10^{-6}
$FeSO_4 \cdot 7H_2O$*	5.0×10^{-3}
$MnSO_4 \cdot 5H_2O$	1.0×10^{-6}
$(NH_4)_6M_{o7}O_{24} \cdot 4H_2O$	1.0×10^{-6}
$NiCl_2 \cdot 6H_2O$	5.0×10^{-7}
H_2SeO_3	3.0×10^{-5}
$Na_2SiO_3 \cdot 9H_2O$	5.0×10^{-4}
$SnCl_2 \cdot 2H_2O$	5.0×10^{-7}
NH_4VO_3	5.0×10^{-6}
$ZnSO_4 \cdot 7H_2O$*	5.0×10^{-4}

6. LIPIDS AND PHOSPHOLIPID PRECURSORS

a) <u>Lipids</u>

- Cholesterol—required by a few cell lines (e.g. NS-1 myeloma) -desmosterol or 7-dehydrocholesterol are better than cholesterol
- Fatty acids—certain cell lines benefit from cis-unsaturated fatty acid, such as oleic acid, linoleic acid and/or arachedonic acid (a precursor for prostaglandin formation)-normally supplied conjugated to serum albumin
- Mammalian cells do not introduce double bonds beyond C_9 into fatty acids. Linoleate (18:2 cis-Δ^9, Δ^{12}) and linolenate (18:3 ces-Δ^9, Δ^{12} Δ^{15}) are thus essential fatty acids.
- Phospholipids—phosphatidyl choline, phosphotidyl ethanolamine,and/or sphingomyelin, which can be added to cell culture medium in the form of liposomes or dissolved in

DMSO, stimulate the growth of some cell lines, cultured under fatty acid and lipid precursor deprived medium

b) Phospholipid precursors

- Choline—precursor for phosphatidyl-choline biosynthesis
- Ethanolamine—precursor for phosphatidyl biosynthesis
- Inositol—precursor for phosphatidyi-inositol biosynthesis—the presence of these compounds can reduce or even eliminate the requirement for complex lipid supplements

7. Nucleic acid (RNA and DNA) precursors

- normally are not essential components of basal media
- a purine source (adenosine or hypoxanthine) together with thymidine is beneficial when folic acid is in short supply (in the case of methotrexate selection) or used inefficiently

C. *NON-NUTRIENT SUBSTANCES*

Nucleotides in basal medium

RNA	DNA
Adenosine	Thymidine
Cytidine	2'deoxyadenosine
Guanosine	2'deoxycytidine
Uridine	2'deoxyguanosin

Most media contain non-nutrient components that can indirectly influence cell behavior by modulating the physiochemical environment of the cell.

1. Antibiotics

- The antibiotics that are used in cell culture are only relatively more

Antibiotics for cell culture

Antibiotic	Recommended	Antibiotic Spectrum
Amphotericin B	2.5 mg/1	Fungi and yeasts
Ampicillin	100 mg/1	Gram-positive and Gram-negative bacteria
Chlortetracycline	5 mg/1	Gram-positive and Gram-negative bacteria
Dihydrostreptomycin	100 mg/1	Gram-positive and Gram-negative bacteria
Gentamicin sulfate	50 mg/1	Gram-positive, Gram-negative bacteria and mycoplasma
Kanamycin sulfate	100 mg/1	Gram-positive, Gram-negative bacteria and mycoplasma
Neomycin sulfate	50 mg/1	Gram-positive and Gram-negative bacteria
Nystatin	50 mg/1 (or 100 U/ml)	Fungi and yeasts
Penicillin G	100 U/ml	Gram-positive bacteria
Polymyxin B sulfate	100 U/ml	Gram-negative bacteria
Spectinomycin	20 mg/1	Gram-positive and Gram-negative bacteria
Streptomycin sulfate	100 mg/1	Gram-positive and Gram-negative bacteria
Tylosin	100 mg/1	Gram-positive bacteria and mycoplasma

inhibitory to bacteria than to cultured cells

- Toxicity testing usually done with fibroblasts grown in media with high serum concentrations
- Serum can protect cells from injurious effects of antibiotics

*Antibiotics should be avoided when possible or, when used, should be cautiously selected for their effects on cell attachment, growth and function and on differentiated cell selection

2. Buffers

Sodium bicarbonate:

- 44mM in DMEM, 14 mM in F12, 26mM in circulatory blood.
- necessary to use 5–10% CO_2 in the incubation chambers; media that contain bicarbonate become alkaline very rapidly due to loss of CO_2 when removed from the incubator:
- The low pKa of bicarbonate (6.1) results in suboptimal buffering throughout the physiological pH range.
- $NaHCO_3$ buffer requires appropriated CO_2 concentrations in the gas phase. The reactions are:

(i) CO_2 dissolves in aqueous solutions. The concentration is described by Henry's Law.

$$CO_2\,(g) \rightleftharpoons CO_2\,(aq)$$

$$P_{CO_2} = H[CO_2](aq) \qquad H\text{: Henry's law constant}$$

(ii) CO_2 in an aqueous solution forms a bicarbonate ion.

$$CO_2\,(aq) + H_2O \rightleftharpoons HCO_3^{-2} + H^+$$

$$Keq = \frac{[HCO_3^{-2}][H^+]}{CO_2\,(aq)} = \frac{[HCO_3^{-2}][H^+]}{P_{CO_2}/H}$$

(iii) The pH of the solution is affected by P_{CO_2}

- From the equation, one can plot the relationship among HCO_3^{-2}, P_{CO_2}, and pH.

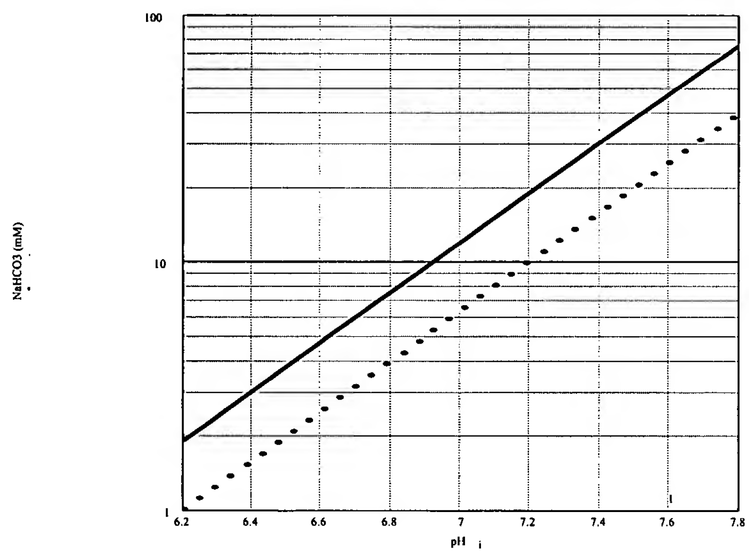


Figure. Relationship between $NaHCO_3$ concentration abd pH. The solid line represents 10% CO_2, the dotted line represents 5% CO_2. For example, at 10 mM $NaHCO_3$, pH is 6.91 for 10% CO_2, 7.20 for 5% CO_2.

Alternative buffers:

- Sodium beta-glycero-phosphate (20 mM) also functions as a detoxifier of ferric chloride hydroxo compounds (i.e., Fe^{+3} chelator)
- Zwitterionic buffers: HEPES (N-2-hydroxyethylpiperazine-N-2-ethane) used between 10–50mM
- Can be growth inhibitor at high concentrations (>25mM) by stimulating cells to make toxic oxygen metabolites
- Requires adjustments of osmolarity (reduction on NaCl levels) when used at high concentrations

Cell culture tested biological buffers

Description	pK_a value at 37° C	ΔpK_a/°C	Anhydrous Mol. Wt.	Working Concentration (mM)	Buffering Range at 37° C
BES	6.90	-0.016	213.2	10 - 20	6.2 - 7.6
BIS-TRIS	6.36	-0.008	209.2	10 - 20	5.7- 7.1
EPPS	7.85	-0.015	132.1	10 - 20	7.1 - 8.5
Glycine 1.0M	9.53	-0.021	75.0	50 - 200	8.7 - 10.7
Glycylglycine	7.95	-0.025	132.1	10 - 20	7.2 - 8.6
HEPES	7.31	-0.014	238.3	10 - 28	6.6 - 8.0
HEPES•Na	7.31	-0.014	260.3	10 - 28	6.6 - 8.0
HEPES 1M	7.31	-0.014	238.3	10 - 28	6.6 - 8.0
MOPS	7.01	-0.008	209.3	10 - 20	7.0 - 8.4
PIPES	6.66	-0.0C9	302.4	10 - 20	6.0 - 7.4
Sodium Bicarbonate	6.28	-0.0055	84.0	2 - 26	5.4 - 6.9
Sodium Bicarbonate 7.5%	6.28	-0.0055	84.0	2 - 36	5.4 - 6 9
TAPSO	7.40	-0.018	259.3	4 - 50	6.8 - 8.0
TES	7.16	-0.020	229.2	10 - 20	6.5 - 7.9
TRICINE	7.80	-0.021	179.2	<50	7.1 - 8.5

3. Phenol Red

- Added as a pH indicator
- Interferes with purification
- Has estrogenic-like activity (MCF-7 cells, C-127 cells)

4. Protective Agents

Compounds which protect cells from damage caused by changes in osmotic pressure, shear, toxic metals and oxidative injury

Many Pluronic surfactants are available. The larger the POE (polyoxyethylene) group, the more hydrophilic the molecule and the greater its detergent-like activity and cell cushioning effects. The larger the POP group (polyoxypropylene) the greater the toxicity and greater anti-foaming ability. Presently, F-68, at a concentration of 0.01–0.1%, provides adequate cell cushioning, but the degree of foaming is high. Therefore, it is desirable to determine if a suitable replacement is available.

The only Pluronics other than F68 investigated that provide suitable growth/productivity are F88 and F77. Growth and productivity are similar to F68 when using 293 cells; however, the degree of foaming is about the same.

Effect of pluronic on cell growth

Type	%POE	%POP	Comments
L-61	10	90	NO GROWTH
L-92	20	80	NO GROWTH
F-68LF	73	27	NO GROWTH
P104	40	60	NO GROWTH
P75	50	50	NO GROWTH
F77	70	30	SIMILAR GROWTH/ PRODUCTIVITY
F88	80	20	SIMILAR GROWTH/ PRODUCTIVITY

Synthetic protective agents used in cell culture

Common name	Chemical identity
Pluronic F68 or F88	Block copolymer glycols of poly(oxyethylene) and poly(oxypropylene) (M.W. ~8350)
PEG	Poly(oxyethylene) glycol (or polyethylene glycol) (M.W. ~20,000)
PVA	Polyvinyl alcohol (M.W. ~20,000)
MC	Methylcelluloses (15 cps methocel) 0.1- 0.2%
CMC, Edifas B50	Sodium carboxymethylcellulose
HES	Hydroxyethyl starch
PVP	Polyvinylpyrrolidone
Haemaccel	Modified gelatin
Dextran	Dextran (M.W. ~78,5000, 20-60 g/ℓ)

a) Structure of Pluronics

$HO \cdot (CH_2 \cdot CH_2 \cdot O)_X$ polyoxyethlene block	$-(CH_2 \cdot CH \cdot O)\gamma$ polyoxypropylene block	$-(CH_2 \cdot CH_2 \cdot O)_\delta \cdot H$ polyoxyethylene block

5. **ANTI-OXIDANTS**

Superoxide radical (O_2-) and hydrogen peroxide (H_20_2) are very reactive with lipids, proteins and DNA; causing damage to cells and media components. These oxygen reduction products are generated during normal respiratory metabolism by xanthine oxidase present in cells and serum and by photo-oxidation of riboflavin-tryptophan

a) Physiologically Relevant Antioxidants

- Vitamin E
- Uric Acid (end product of purine metabolism)
- Taurine (end product of oxidative metabolism of cysteine)
- Bilirubin (end product of breakdown of heme)
- Beta-carotene
- Transferrin
- Ceruloplasmin
- Amino acids
- Selenium

  Selenium-deficient cells are more sensitive to oxygen toxicity (selenium is a cofactor for glutathione peroxidase, an enzyme which helps rremove peroxidases from cells
- Catalase

  Catalase has been specifically shown to improve cloning and growth S49 lymphoid cells and Increase growth and IgG production from hybridoma cell lines
- Superoxide Dismutase
- Reduced glutathione

6. **REDUCING AGENTS**

- ß-mercaptoethanol is most commonly used
- Stimulates cystine uptake (in L-1210 cells) by forming a mixed disulfide
- restores the reduced form of glutathione which helps to prevent peroxide damage
- Stimulates antibody secretion in hybridoma cell cultures

D. *METABOLITES AND CONDITIONING FACTORS*

Produced by cells and secreted into the culture medium

- cannot be ignored, particularly at high cell densities
- may be growth inhibitory or growth stimulatory
- growth inhibitory (e.g., lactate, ammonia C02, peroxides, TGF beta)
- growth stimulatory (e.g., matrix molecules,TGF alpha, IGF-1, IL-2, PdGF)

E. *HIGH MOLECULAR WEIGHT FACTORS (SUPPLEMENTS)*

1. SERUM OR BIOLOGICAL FLUIDS

- Serum is an extremely complex mixture that contains food substances, metabolites, gases, hormones, plasma proteins, substances released from damaged cells (i.e., hemoglobin and growth factors from platelets), and contaminants introduced after blood collection
- Serum has enjoyed almost universal acceptance as a supplement in cell culture medium despite the numerous disadvantages associated with its use (see below)
- For investigative purposes, the costs associated with the use of serum have been minimal
- Fetal bovine serum (FBS) is the most expensive and most widely used serum in animal cell culture due to the fact that it contains higher concentration of growth stimulatory factors and lesser concentrations of growth inhibitory factors than other sera
- Other sera commonly used are human, bovine calf, newborn bovine serum, donor bovine serum and donor horse serum -Biological fluids such as embryo extract, colostrum and lymph have also been used as undefined supplements in cell culture medium

2. HYDOLYZED PROTEINS

- peptones derived from acid or enzyme hydrolysates of casein, gelatin, meat, soy, egg and lactalbumin have been used as supplements in cell culture (see table I A.)
- peptones contain a mixture of amino acids, small peptides, inorganic ions, carbohydrates and vitamins
- ethanolamine was first identified as an important nutrient for cultured cells through fractionation studies of proteose peptone
- for description and utility of peptones in cell culture refer to technical manual "Hydrolyzed Proteins and Sheftone Series" published by Sheffied products (607-334-9951)

a) <u>Functions of serum in cell culture medium</u>

- Modulates physiological properties of medium (viscosity, colloid osmolarity, rate of diffusion)

- Protease inhibitors (alpha 2 macroglobin) neutralize proteases used in trypsinization or produced by cells
- Provides nutrients not present in basal medium (e.g., cholesterol)
- Provides carrier proteins for low molecular weight substances (e.g., transferrin)
- Contains proteins that solubilize nutrients that dissolve poorly (e.g., apolipoprotein)
- Provides factors for cell-substrate attachment (e.g., vitronectin, fibronectin)
- Contains enzymes to convert components to a utilizable or less toxic form
- Supplies "bulk" proteins that prevent non-specific adsorption of critical factors to culture vessel (e.g., serum albumin)
- Binds and/or neutralizes toxic substances in the culture medium (such as detergents)
- Binds and protects essential nutrients, such as fatty acids, that are toxic when present in excessive amounts and releases them slowly in a controlled manner
- Provides hormones and growth factors

b) <u>Disadvantages of serum in cell culture medium</u>

- Can potentially introduce animal virus into cell culture
- May carry antibodies against viruses the source animals have been exposed to. For virus production, such serum containing cross-reacting antibodies will have an adverse effect
- The availability of high quality serum (particularly fetal bovine serum) can become a problem
- Serum may introduce undesirable contaminants into cell cultures (i.e. adventitious agents, antibiotics, proteases)
- The use of serum results in high running costs and unnecessary capital outlay
  - Serum is normally purchased in large lot sizes and stored frozen (- 20 °C) until use. Freezers cost money and take up space.
  - Serum lots must be pretested for growth promoting activity. This demands repeated tedious and time wasting pretest procedures.
  - Serum adulteration of product-rich conditioned medium increases the expense of downstream processing
  - Serum can make the characterization of the final product labor intensive

3. SUPPLEMENTS TO SERUM-FREE MEDIUM

1976 Sato and associates published the first of a series of papers to demonstrate the replacement of serum by supplying mixtures of defined or partially defined supplements (these may or may not

be present in serum), and that different cell lines require different mixtures of these supplements. A typical serum-free medium contains supplements from one or more of the following classes:

- fat soluble and water soluble hormones
- growth factors, lymphokines or cytokines
- transport proteins
- attachment protein
- miscellaneous supplements

a) <u>Factors that effect the composition of a serum free medium</u>

- cell type and/or clone
- transformed or nontransformed phenotype
- basal medium employed
- substrate composition (if required)
- cell density
- physiochemical environment

4. SUPPLEMENTS USED IN ALMOST ALL SERUM-FREE MEDIA

a) <u>Insulin</u>

0.1–10 μg/ml

- available in both glandular (bovine) and human recombinant forms
- available as sodium (soluble in water) and zinc (soluble in HCl) crystals
- relatively stable in tissue culture medium-moderate interspecific potency
- at high doses, may mimic action of insulin-like growth factors

b) <u>transferring</u>

1–30 μg/ml

- requires iron for biological activity
- low interspecific potency; for human and rodent cell lines human>porcine>equine>bovine
- can be replaced by other iron binding protein (i.e. hemoglobin), ferric iron chelators and in some cases by ferrous sulfate
- at high doses, may chelate deleterious trace metals

- purchased from Cohn IV and, unless highly purified, may contain IgG and insulin-like growth factors

c) <u>lipid carrier molecules such as serum albumin</u>

0.1–5 mg/ml

- high interspecific potency
- fatty acid compostion and content depends on method of preparation and species
- most defined medium use fatty acid-free albumin coupled to specific fatty acids, particularly oleic acid or linoleic acid

Fatty acid (mg/g BSA protein) present in BSA

	Cohn fraction V powder	Standard powder	Reagent pure powder
8:0 (Caprylic or Octanoic)	—	0.82	0.04
10:0 (Decanoic or Capric	—	0.09	—
12:0 (Laurie)	—	—	—
14:0 (Myristic)	—	—	—
*16:0 (Palmitic)	0.37	0.15	0.008
*16:1 (Palmitoleic)	0.05	0.015	0.006
*18.0 (Stearic)	0.50	0.017	0.002
*18:1 (Oleic)	0.37	0.077	—
*18:2 + 20:0 (Linoleic) + (Arachidic)	0.13	0.052	—

d) <u>Lipid supplement</u>

- If serum albumin (primarily BSA) is not used in a serum-free medium, lipid supplement is then needed except in some cases in which only fatty acids and ethanolamine are added.

Examples of lipid supplement

- lipoproteins -plasma or serum derived
  - molecules which consist of a neutral lipid core (mainly cholesterol ester and triglyceride) surrounded by a complex of specific apoproteins and phosphlipids (phosphotidyl choline and sphingomyelin) held together by non-covalent bonds
  - bovine lipoproteins mainly HDL type -human lipoproteins mainly LDL type -may influence cell behavior in multiple ways depending on the cell type and serum-free culture environment
- lipid emulsions and dispersions
  - natural lipids (soybean lipid, cod liver oil, sunflower oil)
  - chemically defined lipid mixtures (fatty acids, phospholipids cholesterol)

- emulsifying agents include PLURONIC F68 and TWEEN 80

Lipid supplements in cell culture

Supplements	Suppliers	Contents
Nuserum	Collaborative Research	Serum replacement formulation
CPSR-1	Sigma	Serum replacement formulation
CPSR-3	Sigma	Serum replacement formulation
Chemically Defined	Gibco	Mixture of fatty acids, cholesterol, and phospholipids
Lipid Concentrate Intralipids	Kabi Bitrum, Inc.	Cholesterol-containing non proteineous lipid emulsion
Ex-Cyte VLE	Miles	Low protein aqueous lipids

- Most invertebrate and vertebrate species are not capable of essential fatty acid synthesis and have a very limited capacity for fatty acid desaturation and elongation.
- Transformed cells have fewer essential lipid requirements than normal cells.

Nutrients known to be required by cells in culture (by G. Sato)

Nutrient	Requirement	Special Comments
Sugars		
Glucose	most cells	Can be replaced by fructose, mannose, maltose or galactose for some cells.
Pyruvate	some cells	Required by some mouse embryonic cells.
Amino acids		
Isoleucine	all cells	Early development of mouse embryos, up to and including trophoblast outgrowth, requires all these amino acids with the exception of isoleucine.
Leucine		
Lysine		
Methionine		
Phenylalanine		
Threonine		
Tryptophan		
Valine		
Arginine		
Cyst(e)ine		
Glutamine		
Histidine		
Tyrosine		
Serine	some cells	Some are required by particular cell types. In addition, all appear to be necessary for growth at low density.
Alanine		
Aspartic acid		
Glycine		
Glutamic acid		
Proline		
Vitamins		
Biotin	all cells	
**Folic acid		Can be replaced by a combination of glycine, purine and thymidine
**Niacinamide		
Pantothenic acid		
**Pyridoxine		In one case can be replaced by six non-essential amino acids.
Riboflavin		
Thiamin		
B_{12}	some cells	Shown to affect fatty acid metabolism.
Ascorbic acid		

Nutrients known to be required by cells in culture (continued)

Nutrient	Requirement	Special Comments
Vitamins A, D, & K	none	Appear not to be essential for the cells that have been carefully studied in serum-free media.
Vitamin E	some cells	Does not affect cell growth but is required for certain cell functions.
***Retinoic acid	↓	Affects both growth and differentiation
Fatty acids		
Linoleic and Oleic acids	some cells	Sometimes required in combination with fatty acid-free serum albumin.
Cholesterol	some cells	Required by primary diploid human fibroblasts and a cell line of porcine kidney organ.

Transport and carrier proteins

Transport proteins	Source	Structure	Effects
Serum albumin	Plasma	1-chain (MW=68000)	Supplies free fatty acids Detoxifyer Contains trace elements
Transferrin	Plasma	1-chain (MW=77000)	Supplies iron detoxifyer
High density lipoprotein (LDL)	Plasma	Particle (multiple protein subunit)	Accepts end transports cholesterol end cholesterol esters
Low density lipoprotein (LDL)	Plasma	Particle (Apo B)	Transports cholesterol and cholesterol esters
Trenscobalamin	Plasma		Binds vitamin B_{12}
Ceruloplasmin	Plasma	1-chain (MW=135000)	Binds copper
Hemoglobin	Red cells	4 subunits (MW~65000)	Transports O_2

Adhesion molecules used for cell culture

Adhesion proteins	Source	Structure	Effects
Fibronectin	Plasma, cell lines	Dimer (MW=220000)	Promotes attachment growth of mesenchymelly derived cells
Laminin	Extracellular matrix	2 subunits (MW=900000)	Promotes attachment and growth of ectodermally and endodermally derived cells
Collagens (I-IV)	Skin, extracellular matrix, placenta	1-3 subunits	Promotes attacment and growth either directly or through the binding of other adhesion proteins
Vitronectin	Plasma	MW=70000	Promotes attachment and growth of a variety of cell types
Fetuin	FBS	Alpha 1-globulin	Promotes attachment of cels to glass and plastic
Poly-d-lysine	Synthetic	Polymer (MW=30000-70000)	Promotes attachment of many cell types (even in the presence of serum)

Commonly used growth factors and cytokines

Lymphokines/cytokines	Source	Structure	Effects
Interleukin 1	Macrophage	Protein (MW~17000)	Stimulates T-cell growth
Interleukin 2	T-cells	Protein (MW~15000)	Stimulates T-cells, natural killer cells and TIL cells
Interleukin 3	T-lymphoma	Protein	Stimulates stem cell growth and differentiation
Interleukin 4	T-cells	Protein (MW~20000)	Stimulates beta and T growth
Interleukin 5	T-cells	Protein	Replaces T-cell in antibody response
Interleukin 6 (beta$_2$-interferon)	Monocytes and endothelial cells	Protein (MW~26000)	Stimulates hybridoma cloning efficiency; stimulates beta-cell growth
Erythropoietin	Kidney	Protein (MW~40–45000)	Stimulates growth and differentiation of red cell precursors
Gronulocyte Macrophagecolony stimulating factor (CM-CSF)	T-cells, fibroblasts	Protein (MW~23000)	Stimulates granulocyte and macrophange cell growth and differentiation
Macrophage Colony Stimulating Factor (M-CSF)	Fibroblasts	Protein (MW~70000)	Stimulates macrophage growth and differentiation
Tumor Necrosis Factor	Macrophages	Protein (MW~17000)	Stimulates growth of normal human fibroblasts

Other less-frequently used supplements

Supplements	Source	Structure	Effects
Heparin	Intestinal mucosa	Mucopoly-saccharide MW=6000 20000	Potentiates the effects of FGFs
HPSTI (human pancreatic secretory trypsin inhibitor)	Pancreas urine plasma	Protein MW=6200	Stimulates growth of endothelial cells
H1-30 (urinary proteinase inhibitor)	Plasma urine	Glycoprotein MW-27000	Stimulates growth of endothelial cells
Thrombin	Plasma	2 subunit protein	Stimulated growth of fibroblasts
Cholerataxin	Vibreo cholerae	2 subunit protein	Needed for the growth of some cell types

IV. SPECIAL PURPOSE MEDIA

A. Media design for suspension culture of anchorage-preferred cell lines

Commercially relevant cell lines of this type include BHK, CHO and 293

- Requirement for additional growth factor supplements
  - for some cells the cell-substrate interaction can reduce or even obviate the requirement for certain polypeptide growth factors (e.g. PDGF and FGF); for suspension growth, these factors must be added as supplements to the medium or, if the cells produce them, the cell density must be maintained at critical levels
  - certain growth factors can bind to the substratum and remain biologically active (insulin to plastic, beta-TGF/Gi to fibronectin, PDGF to plastic)
  - some growth factors are more potent when bound to matrix molecules (FGF to heparin)
- Reducing cell-cell and cell-vessel interaction
  - cells clumping and cells sticking to vessel surfaces can be a major problem when these types of cells are initially transferred from attached culture to suspension . This problem can be partially corrected by the following media design modifications:
    - Pluronic F68 (0.01–0.1%)
    - Heparin (10–100 ug/mi)
    - Replacing FBS with horse or calf serum
    - Lowering Ca^{+2} (~0.1 mM)
    - Dextran sulfate
    - Increasing albumin concentration in serum-free medium

B. *Media for suspension culture of insect cell lines*

- Excellent technicial information available from GIBCO/BRL, JRH Biosciences and Sigma
- Most commonly used basal medium for culturing insect cell lines are
  - dipteran cells- Mitsuhashi and Maramorosch MM medium
  - drosophila cells- M3 medium
  - lepidopteran cells- Graces medium and modifications (IPL-41)
- these media usually require serum, peptone, and/or defined protein supplements to support growth.
- glucose and fructose are preferentially utilized as energy sources.
- glutamine utilization or breakdown do not appear to be rate limiting.
- 14 amino acids are essential for growth; alanine, aspartic acid, glutamic acid, and glycine are non-essential
- sterols (i.e. cholesterol) are considered essential for growth. Unlike most vertebrate cells, insect cells cannot synthesize sterol.
- high shear sensitivity and oxygen demand requires the use of protective agents such as methyl-cellulose, PVP-40 or Pluronic polyols.

C. *Maintenance media*

- Product production and secretion by mammalian cell lines may be optimal only when cells are in an actively growing state, or in a non-proliferative or slowly dividing state
- In the former case, the culture media must be designed to simultaneously support both growth and productivity at their maximum rates
  - for "normal" cells this usually means the addition on high concentrations of serum or costly growth factors
  - for transformed cells (i.e. hybridomas, CHO, etc.) this usually means the addition of lower concentrations of serum or less costly, non-growth factor supplements
- In the latter case, the culture media (and bioreactor) must be designed to arrest or slow cell growth and concurrently support survival and maximize productivity
  - the design of medium to growth-arrest cells also depends on whether the cells are "normal" or transformed
  - normal cells usually growth-arrest upon depletion in serum growth factors, deprivation of essential nutrients such as various amino acids (i.e. isoleucine), Ca^{+2} ion, glucose or phosphatate ion or addition of compounds that promote cellular differentiation

    - transformed cells often growth-arrest due to essential nutrient deprivation or addition of pro-differentiation compounds. However, such cells usually do not go into a quiescent state when depleted of serum growth factors, and as a result they die quite rapidly
    - survival requirements are generally less complex than those for growth. These depend, in part, on cell density, the nature of the substrate and presence of toxic contaminants. In many cases, survival (or maintenance) can be sustained in basal medium with minimal amounts of serum or supplements.
- In the absence of cell division, some media components (i.e., fat soluble vitamins) turn over very slowly. To demonstrate a requirement for such components under these conditions may take days or weeks of continuous culture.

V. MEDIUM FOR INDUSTRIAL CELL CULTURE

The strife to rid cell culture of serum and additives of animal origin has begun to bear fruit as serum-free culture has been in common industrial practice for a number of years for recombinant products. CHO cells were serially propagated in serum-free medium containing recombinant human insulin as the only medium protein component up to a 8000 liter stirred tank bioreactor (Keen and Rapson 1995). Although the original adaptation to serum-free conditions was performed with cells adherent to flasks, subsequent subculture allowed cells to eventually grow as small clumps. Protein-free and cholesterol free medium has been developed to cultivate both hybridomas and glutamine synthetase (GS) transfected myeloma cell line NS0 (which is normally a cholesterol auxotroph) for the production of humanized antibodies (Keen and Hale 1996). In other work, it has been shown that NS0 clones can be selected using classical microbial techniques to correct for perceived auxotrophies such as cholesterol (Birch, Boranston et al. 1994).

The adaptation of cells to serum- or protein-free cultivation is often clonally specific, usually requiring medium reformulation and/or adaptation for each new clone. It was recently shown possible to adapt wild type host CHO cells to serum-free cultivation before transfection (Sinacore, Charlebois et al. 1996). The resulting (recombinant) clones retained the ability to grow in serum-free suspension culture even after gene amplification and the products produced were biochemically and structurally similar to their counterparts derived from unadapted host cells.

In the past few years, serum-free medium is becoming the norm for industrial cell culture for rDNA protein production. Newly developed processes are even free of animal components, although in the manufacturing of many viral vaccines, bovine serum is still used, at least in cell cultivation if not in virus production stage. In tissue engineering, though, especially where primary differentiated cells are used, the elimination of animal serum from culture medium takes much more effort. Usually, the first step in eliminating

serum is to develop a reduced-serum medium. Only after that are the cells adapted to serum-free, animal-component free, or even chemically defined medium.

A. *Reduced serum medium*

- Improve on basal medium to contain all essential and many non-essential nutrients, including all raw materials needed for synthesis of new cell substrates for energy metabolism, vitamins, trace elements and bulk inorganic ions
- Supplement sera with supplements (i.e., peptones) to augment the levels of some essential components (sera extenders)
  - Transferrin may be a limiting factor when human/rodent cells are cultured in calf and fetal bovine sera
  - Employing a more physiological substrate (i.e., extracellular matrix) for cell attachment can help reduce serum requirement
  - Maintaining higher cell densities can lead to reduction in serum requirement due to "autocrine" or "conditioning" factors

B. *Serum-free media*

- Serum-free medium consists of nutritionally complete basal medium supplemented with an empirically determined mixture of hormones, growth factors, attachment factors, attachment proteins and binding proteins (these mixtures are available commercially and are referred to as serum replacements).
- Limitations of existing serum-free media for scale-up:
  - Serum-free media may not work for purposes very different from the one for which they were originally developed; reactor conditions are quite different from those of T-flasks
  - Serum-free media are usually specific for a specific cell type-no single medium is suitable for all cell lines or even clones derived from the same parent cell line
  - Serum-free media are, in may cases, suboptimal in their ability to promote cell growth and/or function
  - Serum-free media are constructed from components that many times are either too costly for scale-up or commercially unavailable
  - A number of "serum-free' media developed in the past contain large amounts of one or more partially undefined supplements (i.e. serum albumin, fetuin, neuronal extract) which may lead to problems with product stability or downstream processing

- Despite the above problems, serum-free media have successfully been used in the large scale cultivation of animal cells, including hybridomas and lymphoblasts cell lines, recombinant CHO cells, Bowes melanoma, recombinant C-127 cells and spodoptera frugiperda cells

C. *Chemically-defined medium*

- Chemically defined media exists for a number of cell lines, but at present, these are limited in application in large scale animal cell culture except for hybridoma and myeloma cells
- Progress in this area will likely be accelerated by
  - an urgency to demonstrate control over all aspects of production and downstream processing for licensing by the FDA
  - the likely availability of recombinantly produced (in E. coli) tissue culture supplements (i.e. Insulin, EGF)
  - genetic engineering of cells to produce their own growth factors
  - development of small, synthetic peptides that can mimic the action of the larger, naturally occurring protein (i.e., RDG sequence)
  - design of better, more physiological support matrices
  - acceptance of continuous bioreactor systems and the operation of these systems in a "maintenance" mode

VI. MEDIUM COMPOSITION TEMPLATES

For the composition of commonly-used basal media and supplements in spreadsheets, see the Medium Composition spreadsheet (*MedComp.xls)*.

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